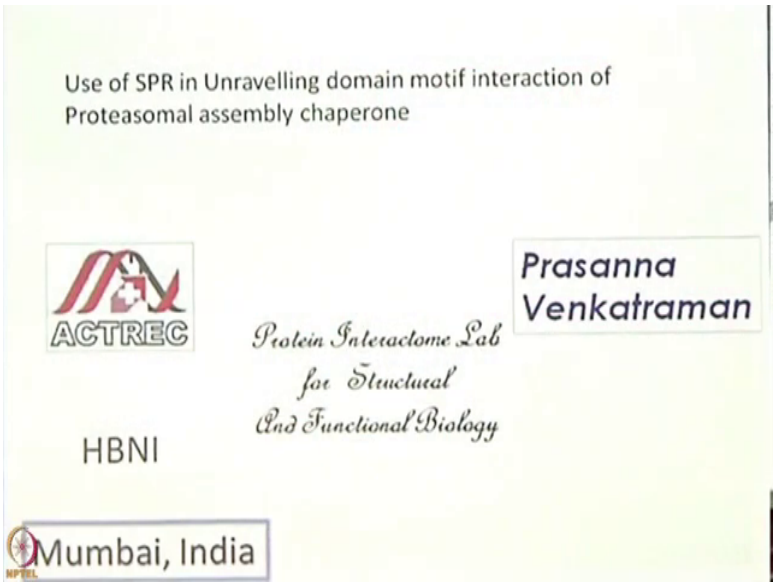
Interactomics: Basics and Applications
Prof. Sanjeeva Srivastava
Dr. Prasanna Venkatraman
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay

Lecture – 36
Use of SPR in Unravelling Domain Motif Interaction of Proteasomal Assembly Chaperones

Today's lecture will be conducted by Dr. Prasana Venkatraman, who is principle investigator and scientist at Actrec Mumbai. Dr. Prasanna's lab is interested in general understanding of mechanism of cellular homeostasis both in health and disease. Her group is trying to develop a model system to navigate through the various steps involved in proteasomal degradation. She is also interested to understand how the communication between substrates and the proteasome; then translates into downstream events like unwinding of the polypeptide chain and its subsequent degradation.

In today's lecture Dr. Prasanna will further explain, the surface plasmon resonance SPR technology to know why and when it is applied to study the protein-protein interactions. So, let me welcome Dr. Prasanna for today's lecture.

(Refer Slide Time: 01:39)



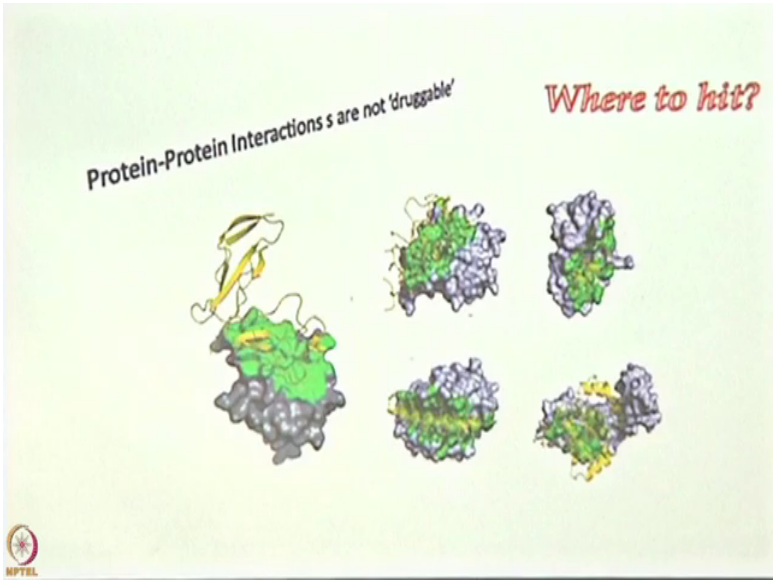
Hi, very good morning. This is the title Use of SPR in Unravelling domain motif interaction its of Proteasomal assembly chaperones. But before directly going into SPR I wanted to give the philosophy of what this domain motif interaction is. Maybe not all of you are familiar with it. And especially in the context of a non-core protein where instead of one on one protein interactions, the protein interaction exists as a network which changes from the normal to the cancer cells, you need to understand how would one look at from a network perspective.

And even if you looked at the network perspective, at the end of the day you want to inhibit these protein-protein interactions with small molecules. So, how do you get to the details of it from a global perspective and you narrow down it to single protein-protein interactions. Or destabilize a network of interactions because you have a common principle that drives this

interaction. And, that is basically the domain motif interaction concept that can be extrapolated in the context of a protein interaction network.

I will show you one example I do not have time to go for two both. And then in the end I will tell you how having done different techniques, I will ask you a few questions of how SPR is going to help us in taking it to the next step.

(Refer Slide Time: 02:48)



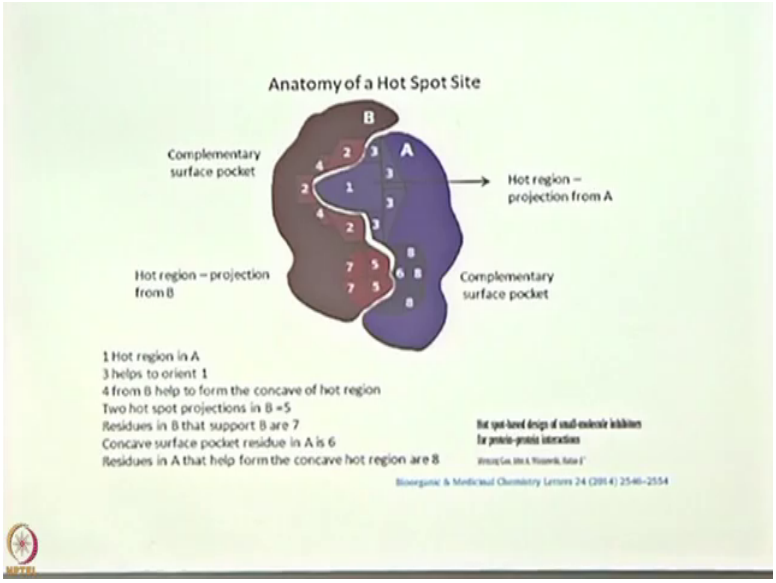
So, these are some of the structures of protein protein two proteins interacting it does not matter what they are. And those of you were very familiar with small molecule protein interactions in terms of a substrate enzyme interactions.

You know that they will occupy a very small site active site is very small and the molecules that bind to the active sites are very small. So, you burry only a small surface region like a

600 Armstrong square region when the small molecule binds with a protein. Whereas, if another protein interacts with a protein then they burry really large surface areas which can go up to 400 500 Armstrong square of buried surface region, that is what contributes to a high affinity of interaction.

And because you have a large surface area that covers the interface, you cannot find a principle that will inhibit this interaction right. So, this seems so, complex and they are enveloped against each other over a very large surface area for you to pinpoint that there is a specific region that, I would like to target with a small molecule becomes difficult and that is why people say these are undruggable. Now, if they are undruggable are they really so, and can you convert them into druggable targets, using principles of biophysics and biochemistry.

(Refer Slide Time: 04:08)

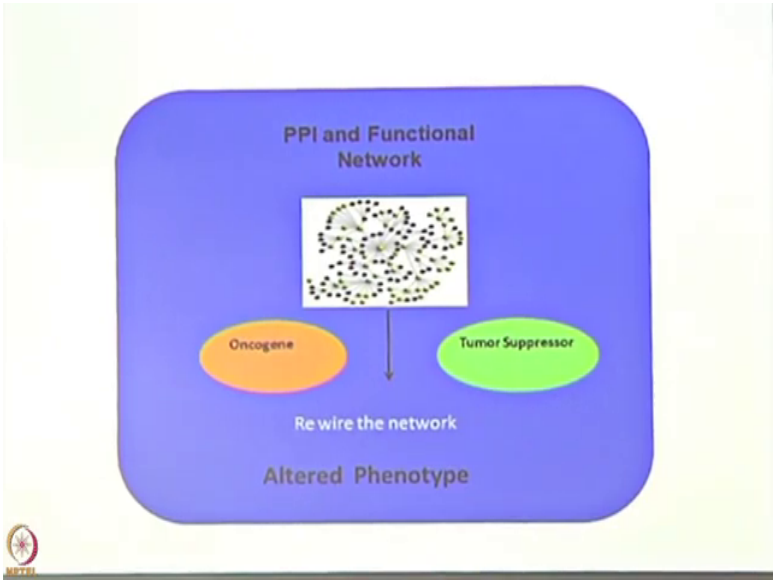


Now, long ago people worked on these protein-protein interaction dissected them and this is your reductionist picture of a protein-protein interaction. Even though they envelope over really large surface area, you can see this protrusion that is a knob that is sitting into this hole right. And, if you mutate any of these residues within this region which is normally 1 or 2 this will fall apart.

So, despite a large surface area that dictates the interaction, the key interactions are driven by very few residues at the interface and these are called as hotspots. In many cases it can be a single amino acid that is dictating this bulk of the binding energy. And if you make the mutation in this residue, you will weaken this interaction that they will not be able to associate with each other. So, this is the concept of a hotspot interaction and this has been increasingly shown to be true in a number of interactions, where you are able to identify such a key residue that is responsible for interaction.

It seems easy it is not trivial, but it is being increasingly observed that this can be true for many many protein interactions.

(Refer Slide Time: 05:15)

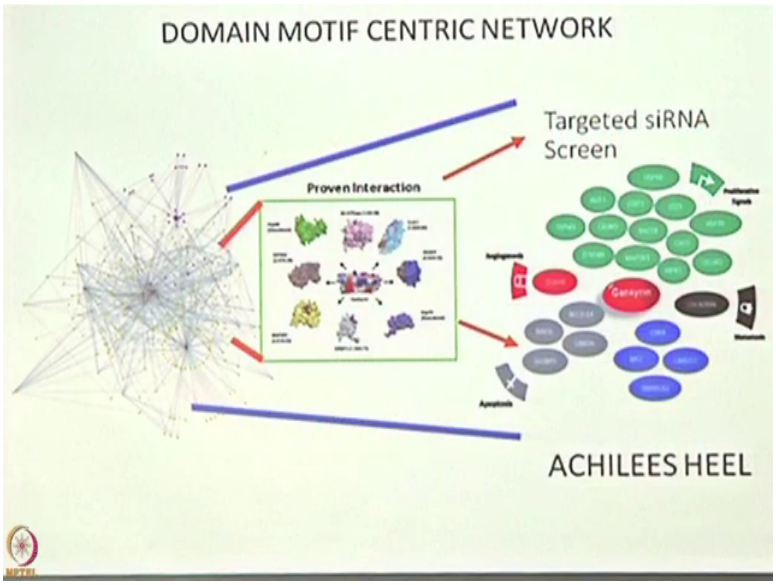


So, I said that no longer can we think of single protein-protein interaction, we love to view protein interactions as a network weather at different hubs with their own islands of interactions. There are unique edges and these edges are connecting different hubs as well. So, this is the kind of interaction that exists in a normal cell and if you had a oncogene or a tumor suppressor, because of their levels mutations they can what they can do is that they can rewire the network; because protein interactions are primarily determined by affinity.

So, if you have over expression or a mutation that can affect this binding, you will deregulate this network. Some of these interactions may get strengthened they just may be stronger some of these interactions get weaker and that is how you rewire the network. And that is the difference between a normal and a cancer cell. Now, how do you view this how do you view

this in the concept of protein-protein interaction? How do you study it using biophysical techniques such as SPR.

(Refer Slide Time: 06:16)



So, as I said this is the network this is made of multiple interactions. So, what you are trying to do is that, can I find a sub network within this? That I can describe in terms of known structural and by physical principles. So, if you consider that there is a domain and a motive and these are interacting and this interaction is a conserved interface what most of the time happens is that, hubs generally borrow this common interface. For example, I have residue something like evd, which will come in the context later there are four residues that are at the interface in this complex.

The hub will borrow this interaction interface use its site to interact with multiple proteins which carry the same motive. Now this proteins need not be homologous they need not have

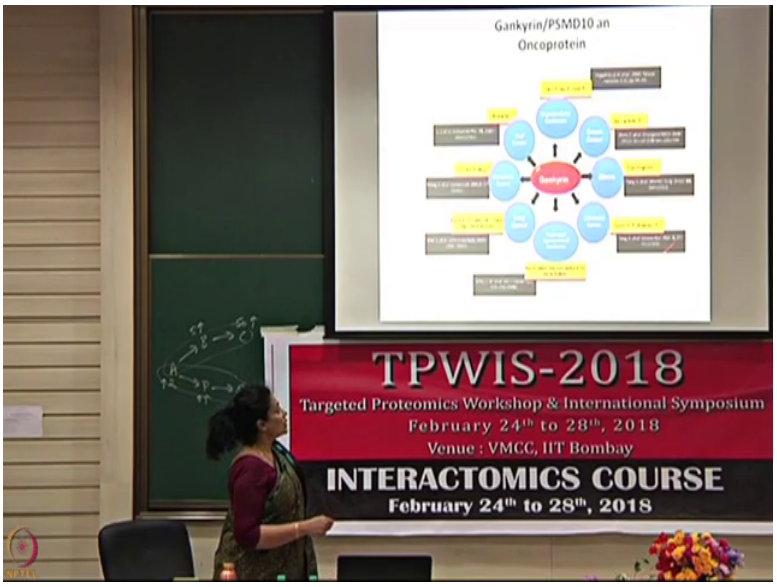
carry any sequence similarity, but they happen to carry the short sequence within them, what are called as short linear sequence motive. And if you have this conserved across multiple proteins these would interact with this hub. So, now, you have a hub centric network dictated by a single motive.

And say for example, I understood what is the residue that is contributing to this interaction among the EVD or maybe just these 3 residues are really really important for interaction I mutated I destabilized. So, I destabilized not just a single interaction I destabilized multiple interactions got the concept. So, this is how you go from principles of domain motif interaction to the network. And then, you come to the reductionist approach of destabilizing the network using the very same principles that is what we had employed in our study. To look at Gam Karen its a proteasomal chaperone assembly chaperone as well as an as a oncoprotein.

Then what you could do is that, to know which one of them may be functionally relevant you can couple it to a genomic approach where you use siRNA to knock out each of these genes and see if pick up really the functionally relevant ones or which one actually dictates cell fate, malignancy, metastasis. And then you could go for that particular interaction in the cancer type that you have.

So, if this is the global picture of what our concept is our philosophy is and we call this as trying to find out the Achilles heel in cancer.

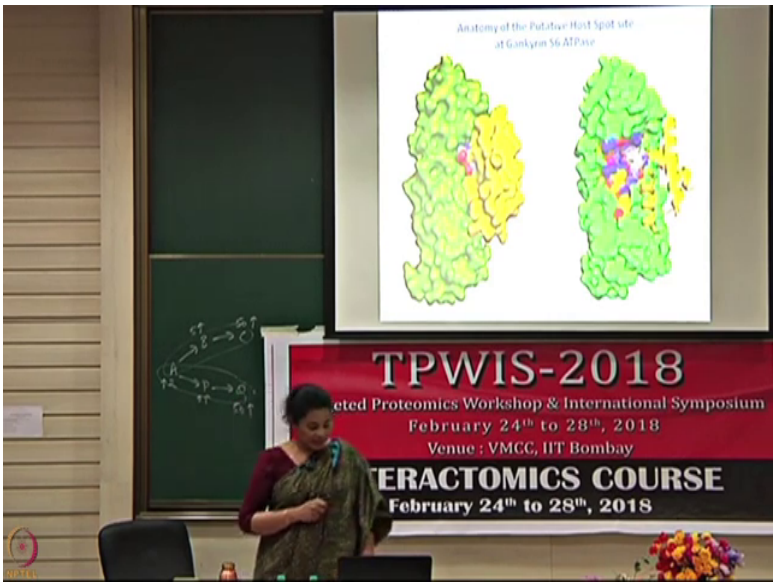
(Refer Slide Time: 08:39)



So, now let us go to the directly to the example. So, this is PSMD10 or Gankyrin that is a proteasomal assembly chaperone do not worry too much about it; proteasomal is like huge machinery for it to work. It needs to be assembled from different protein subunits and these chaperones help in the assembly. And what happens is that Gankyrin also turned out to be an oncoprotein which is overexpressed in multiple cancers.

And it is as you can see in this it is involved in a plethora of cancers and plethora of hallmark cancer properties.

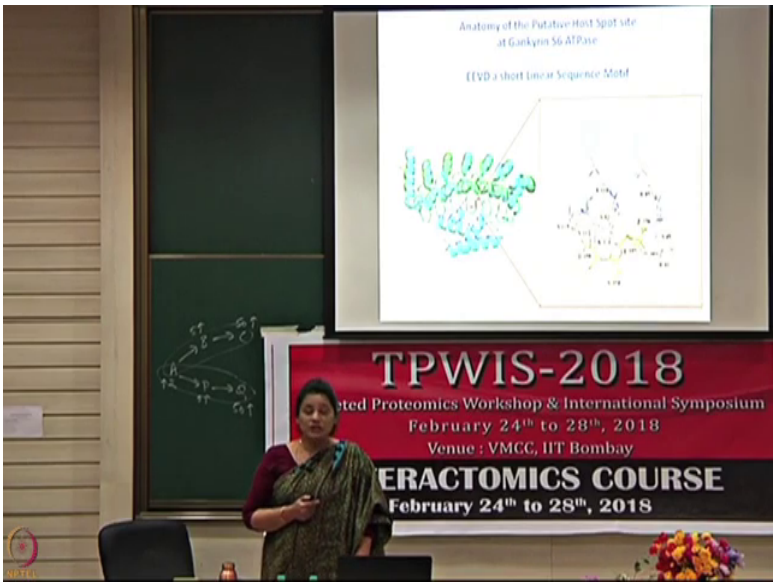
(Refer Slide Time: 09:09)



What was available to us was, a crystal structure of Gankyrin and one of the assemble assembly component, which is ATPase of the proteasome. And you see that there is an interaction between Gankyrin and S6 ATPase through similar to the knob and the protrusion that I showed you earlier.

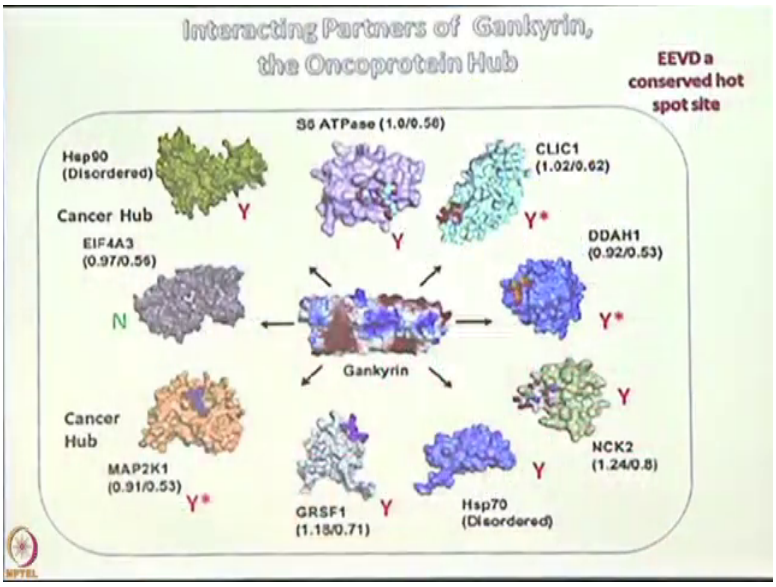
So, we recognize this as a hotspot the crystal structure is available, but we recognize this as a potential hot spot. We looked at residues that are there and we found that a EEVD ok.

(Refer Slide Time: 09:39)



From the S6 ATPase protrudes into the Gankyrin surface. And if there are proteins which have EVD on the surface of on its surface, they are likely to interact with Gankyrin that is the prediction a bioinformatics prediction.

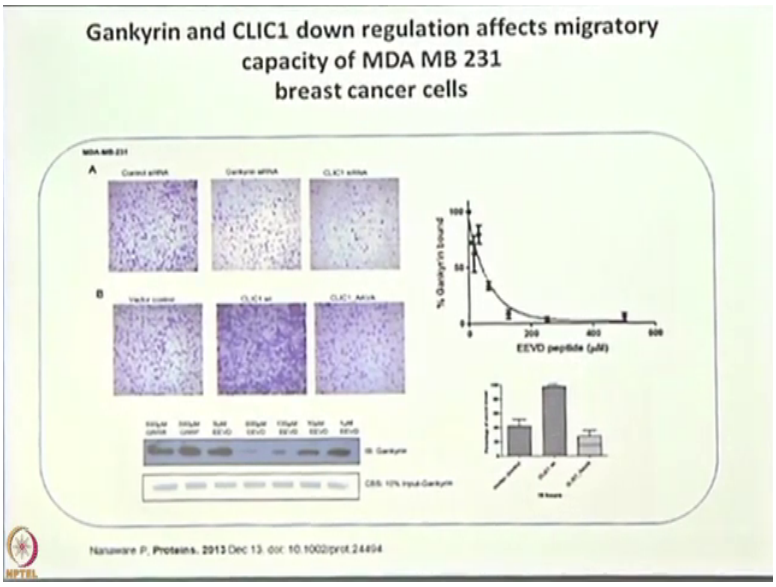
(Refer Slide Time: 10:00)



And we had some 32 proteins for which the crystal structures were available. So, we know that these are in the accessible regions of the protein and we asked tested 8 of them as to how many of them would interact. And then except for this one protein we found all of these that we predicted interact with Gankyrin experimentally. And if you mutate the EVD here you lose interaction this is already published.

And as you can see that seven of them drives this interaction through EVD. So, this is a subnetwork within the Gankyrin EVD interaction.

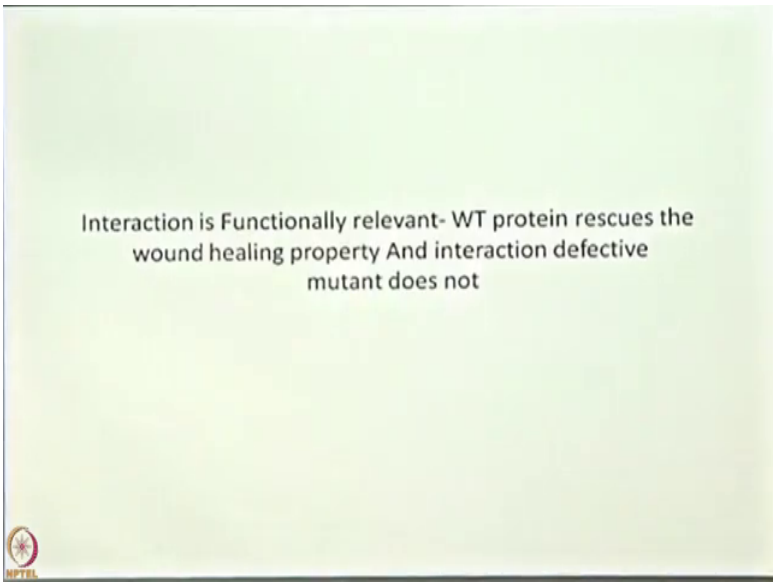
(Refer Slide Time: 10:31)



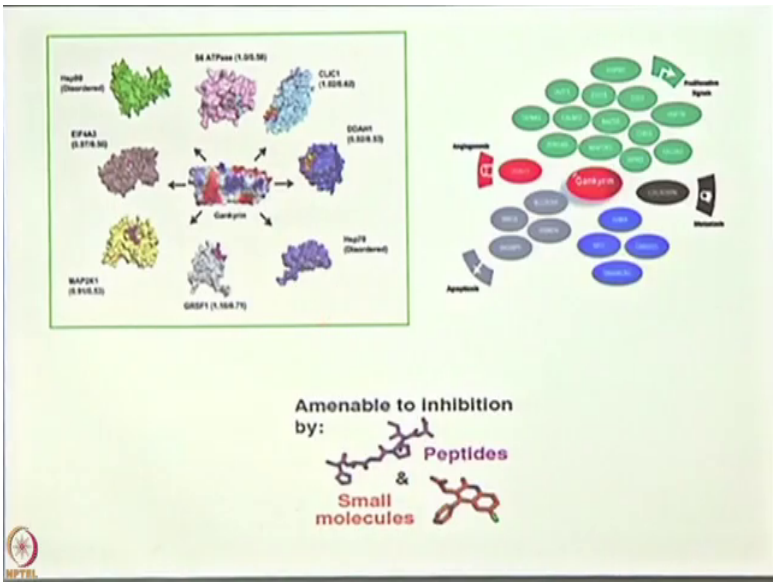
Now, is it functionally relevant? So, you have to demonstrate that this physical interaction makes sense. We did a series of experiments where you knock out the one of the interacting partner. And then you overexpress a wild type or the mutant the wild type is able to rescue the phenotype the mutant is unable to rescue the phenotype. The peptide alone a short EVD peptide is able to inhibit this interaction and that can be quantitated and you can get an IC 50 of about 50 micromolar.

So, that is important right, when you are saying this peptide is responsible the mutation is one way of doing it. And throwing in the peptide to inhibit the interaction is another way of establishing that.

(Refer Slide Time: 11:07)



(Refer Slide Time: 11:09)

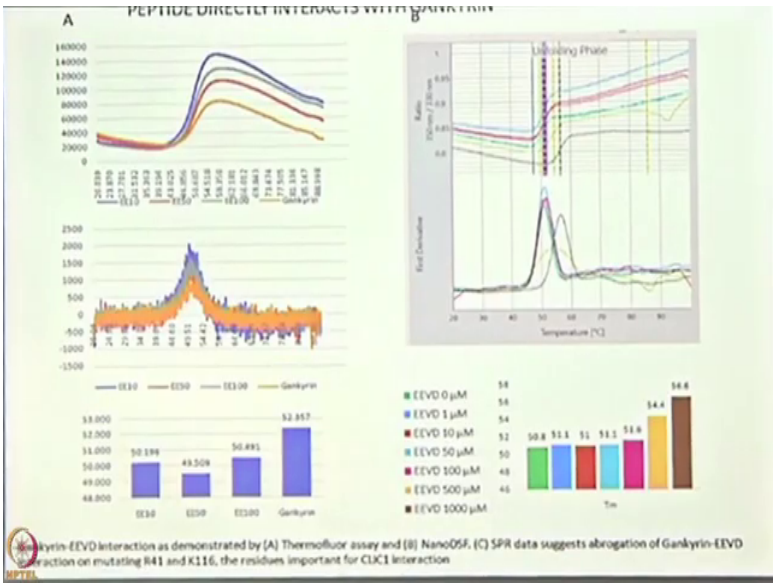


So, this we did and we know that we can go this route to find the achilees heel and you can see that many of these proteins are involved in many cellular activities; like angiogenesis apoptosis proliferation and metastasis.

So, we are looking at the functional network of Gankyrin, through this domain motif interaction and the conserved hot spot site surface why is it important? Because now we have reduced the surface like a small molecule EVD is now like a small molecule, you can perturb it by peptide derivatives or small molecules. You are converting a large surface area now into something that will mimic a small molecule protein interaction.

So, what was non druggable earlier is now becomes druggable; because you are looking at the key interactions that stabilize the complex. And therefore, you can put up them with small molecules and peptide derivatives.

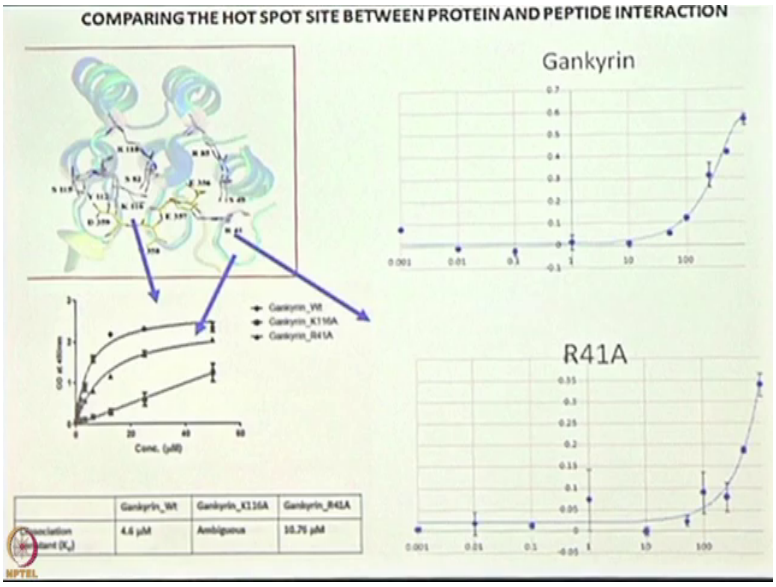
(Refer Slide Time: 12:04)



So, now does the vector I directly interact with the protein. You need to do a series of experiments to establish that the peptide directly interacts. Of course, it interacts because it inhibits the complex, but does it directly interact.

We did a series of experiments where there was a thermal floor assay and there was also a danode yourself, I think you are familiar with it now that the courses have been conducted. So, you can look at these and then we established that there is a direct interaction.

(Refer Slide Time: 12:31)



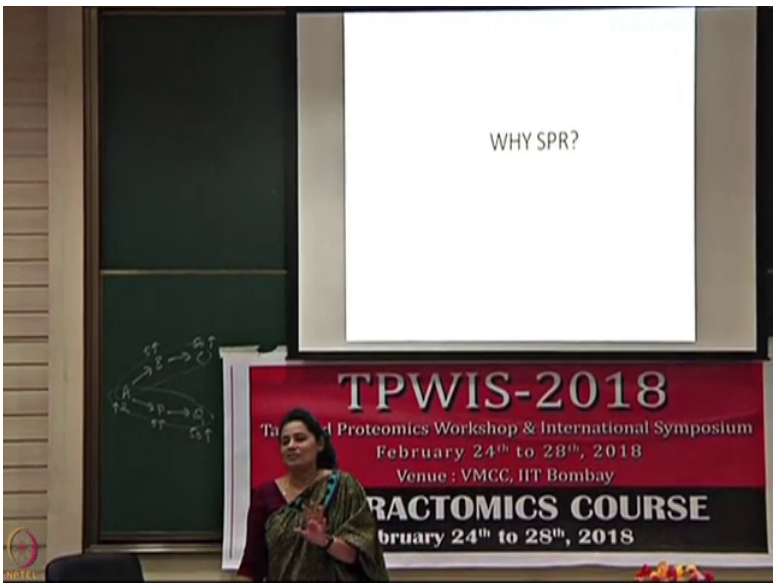
We went back to our old technique of eliezer where you can look at peptide protein interaction only that the peptide is now labelled with a biotin. So, that you can pick it up with a streptavidin alkaline phosphatase. You can see this is the wild type peptide interaction, it does not go on to the saturation there is a still in process we probably have to get new peptide preparation this is hot from the oven.

And, then you can mutate the same residue that we think is important for interaction from the Gankyrin interface. Because you say saw that the S6 ATPase comes with EVD other proteins are coming with EVD in their sequence. Gankyrin uses lysine and lysine 116 and R 41 to interact with the 6 ATPase. We believe that it will use the same residues to interact with other proteins as well as with the peptide are we right. Here is an interaction with click one of the

interacting partners which we suspected would interact with the EVD. Here you can see when we make a lysine or an arginine mutation you abrogate interaction.

You do that similarly for the peptide and I show direct binding of the peptide to the protein from the other essays. So, we have now narrowed down to the peptide protein interaction using this short motive.

(Refer Slide Time: 13:43)



Now, that we have all these answers we know the peptide binds. If peptide binds at the intended place it is able to compete with the protein. So, why should we do SPR? That is a question to you why do we need SPR? We also have a crystal structure of the protein by the way its published, but we can solve the structure we are soaking it with the peptide to determine that structure that is different. So, if we can go to that extent why do I need SPR?

Student: Kinetics.

Kinetics very good what is kinetics how is kinetics going to help me? Nice kinetics, but why how is it going to help me? Why should it help me? Why should I bother to study kinetics?

Student: To compare the on and off rates.

I should compare the on and off rate very nice, but why should I compare on and off rates? Why am I not satisfied with dissociation constant equilibrium dissociation constant kd? What is the relationship of KD and on and off rate? KD is equal to.

Student: K off.

kd off by K on these are rate constants right; they are equated to the equilibrium rate constant, but I equilibrium dissociation constant but why? So, what? So, once you have the ratio what does it mean? The ratio will determine the equilibrium dissociation what does that mean then? So, if I have the same KD what do you think will happen to the on and off rate? I have so 4 molecules I have the same KD for them.

Student: On and off rates may vary.

Very good that is the reason to do SPR ok, but still even if I knew that the on and off rates are different, how does it help me in the next step? How can I use this? How can I apply the SPR derived information which can be given by no other technique right; real time kinetics of interaction is there any other technique that can truly give you the label frees SPR is the one that will give you. So, why should I do it? What am I going to get out of knowing what is the K on and K off?

Student: You get to know the off rate. So, you know the resistance standard will help the (Refer Time: 15:53) you need to know the optimum concentration of the drug (Refer Time: 15:57).

Ok.

Student: Because most of them would have a similar affinity and the different off rates.

Correct. So, how do I engineer it now? If suppose I want to make it more I want to dissociate it slowly or I want to have a rapid association. So, most likely you want to slow the dissociation of a drug right and you do not it to slow because you know.

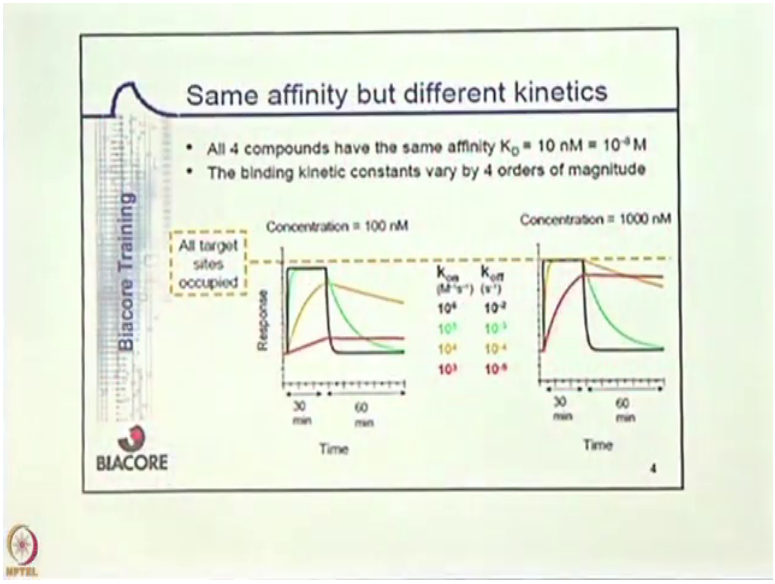
Student: (Refer Time: 16:19).

Exactly. So, what would I do when I look at the structure? Can I can it help me in any way can structure help in any way? So, independently K on and K off I have and I want to improvise this in producing a drug that will compete with this peptide or it will compete with the protein-protein interaction I want a drug. And I want to get the drug small molecule inhibitor which will go into a drug with all the properties that are necessary. So, simple on and off rate will tell me or what should I do.

So, how do I improvise on the drug how do I engineer the drug? Now you alter the pharmacores of these right, you look at the interactions with the protein and then you find out what are the molecules what are the residues that are interacting. Whether it is a hydrogen bond whether it is a salt bridge whether is a hydrophobic interaction. And knowing the residues that are interacting can I now better these and can I engineer a better drug. Or, if I engineer a better drug I am thinking I am engineering a better drug and I come and look at the SPR maybe. I did not achieve the goal I actually made it into a poor a it had a poor outcome as compared to what I expected right.

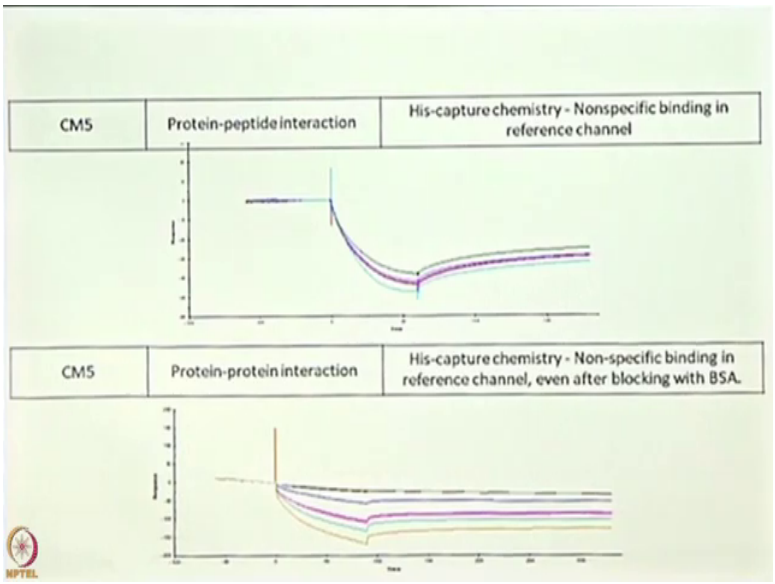
So, you couple the structure guided drug design look by with the SPR on and off rate. You can actually begin to look at how to get the best drug that is possible engineer it with the properties that are favourable for a drug protein interaction that will displace the protein ok.

(Refer Slide Time: 17:57)



So, this is an example where you had the same KD, but different on and off. And as you guys rightly pointed out since the ratio is going to change you can have the difference you can find out the molecules that are different.

(Refer Slide Time: 18:08)

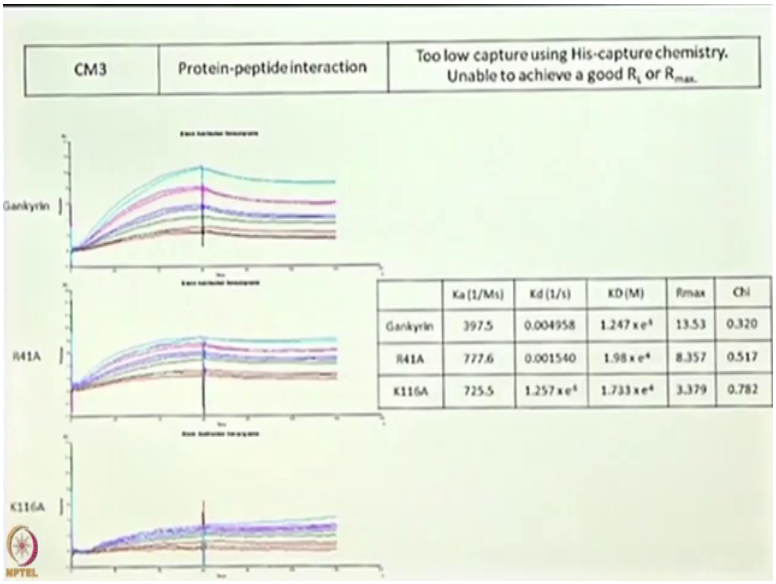


So, you should look at the bio rad bulletin and the GE bulletin to understand these things yeah. So, we started off trying to now we had a fairly good idea of how the peptide is interacting with the protein. And then in the crystals that we soak with the peptide we do get the crystals, but all the crystals that we have diffracted still now have awakened binding set we have not formed the peptide there, but the process is ongoing. So, here you can see that we tried the have you do you know about the s CM 5 chips CM 3 chips?

Yeah. So, these are chips with the Dexter molecules to which the protein binds you can immobilize them yeah so, ok. So, we tried the CM 5 and chips here which is normally used for these kind of studies protein peptide interaction. And we also tried that his capture his beam capture chemistry by having the nickel NTA there on this in all the cases what we found is that there is a nonspecific binding to the reference cell. So, once you subtract you do not get

any interaction. And then nonspecific again in the reference cell even after blocking with the BSA and we have not been able to successfully get the CM 5 working.

(Refer Slide Time: 19:14)



Then we went to CM 3 one of the suggestions because what is the difference between a CM 5 and the CM 3 interaction chips?

Student: (Refer Time: 19:21).

Exactly. So, they do not; so, they have a lesser surface to bind and therefore, you can control your binding your occupancy better and therefore, you are going to avoid this nonspecific binding that is the logic. We did some improvement to this you can see this is the wild type protein and the peptide interaction. And in each case you see that we have done a duplicate

run with a peptide and then you have the R 41 and K 116 A mutants because we know that interferes with the interaction.

You can see they do interfere with the interactions. So, what is your take on these SPR sensograms? Do you like it, do you not like it? Is it ideal is it interpretable? What are the features that you see? What are the features you should see and what are the features that you see?

So, do you know how to interpret a sensogram, what is in the x axis?

Student: Time.

Time what is in the y axis?

Student: (Refer Time: 20:19) RU unit.

RU units and then what is the sensogram telling us?

Student: (Refer Time: 20:25) there is an association phase.

There is an association phase and the dissociation phase great. And what is in between the association and the dissociation phase? So, when do you start dissociating? How do you determine for what time that I need to run the analyte, when do I stop and start dissociation?

Student: (Refer Time: 20:45).

Very critical, can I say arbitrarily I will run it for 10 seconds and I will just wash it off? No. So, what determines it?

Student: Saturation (Refer Time: 20:55).

Saturation. Do we call it a saturation? Saturation by definition is what? Saturation by definition is.

Student: When all the sites are occupied (Refer Time: 21:09).

All the sites are occupied will that happen when you have lower than lower concentration and KD it will not. So, what do we call that as? Equilibrium, yeah. So, you need to achieve equilibrium have you achieved equilibrium here? Maybe, maybe not maybe, but we should try and extend the time for equilibration this you learn by trial and error you fix some time for the different concentrations and as the time goes you improvise on that, but this is not too bad ok.

And what is happening with the dissociation? Is dissociation critical I already got this why do I worry about (Refer Time: 21:49). I just flush get all the analyte out of it should I wait for the dissociation to happen, why is this association important?

Student: All the drug remains (Refer Time: 21:57).

Very nice. So, what does it determine what does dissociation determine here?

Student: The off rate.

Exactly the off rate right that is. So, what is so? In something important about the off rate why should I, if I did not have operate in SPR, I will convert this into a normal equilibrium study where I get the KD values ok; it makes no fun. Unless I get the K off and that comes from the dissociation phase. And how should the dissociation be? Are you happy with this dissociation? Criticize you are happy with the decision why is that?

Student: It is too slow.

Very nice it is very slow right. So, if I if then if I want to keep do you think I will ever achieve complete dissociation in this case?

Student: No.

Why not?

Student: The time given is not enough to dissociate completely.

So, I will keep it for 3 days no ok.

Student: The time (Refer Time: 22:55) which is see which is yeah which is parallel.

Yeah, but imagine over the days it will come like this ok; it will definitely come, but that is not the point. So, how do I now. So, what should I do now? I will not get a good off rate from this. So, what should I do? And without off rate my kinetic estimations are not going to be right.

Why is it why is it K off so important? Why cannot I look at K A which seems very good and if I achieve the equilibrium I should get the K A. What is so, unique about K off? K on as diffusion control if it is diffusional control and there is also it is controlled by another parameter what is the difference between K on and K off?

Student: Concentration.

Excellent. the first one is concentration dependent K off is independent of the concentration; because now I am looking at a complex a b it is there it is not determined by concentration. So, the inherent property of the binding comes from this independent variable that is K off independent constant which is K off. And if I can determine that I my all my kinetic estimates are going to be more or less accurate right.

And imagine I have the right K off, I have KD which is a equilibrium constant I did determined by n number of methods right what can I get? I know K off I know the equilibrium dissociation.

Student: K on.

I can get K on right. I can get a rate constant for association independent of the instrument and can I go back and verify whether I got the same K on or not? Yes right and do you think the KD that I determine by SPR that is a equilibrium dissociation constant is going to be very different from the ones that I determined elsewhere. Say for example, analyzer it should not right. And how do I get an equilibrium constant from the SPR data?

Student: Steady state steady state measurement.

Steady state measurement; how do I do a steady state measurement?

Student: Just like different concentration (Refer Time: 24:55) in different concentration.

Different concentration and what do I plot?

Student: Concentration and time (Refer Time: 25:01).

How does it look like?

Student: Lineweaver Burk plot.

Very nice. So, you guys are experts here. So, then there do I need equilibrium or do I need saturation?

Student: You need equilibrium.

Do I need saturation?

Student: And saturation.

Very nice. So, equilibrium whether it is a kinetic measurement or equilibrium measurement. You need equilibrium and you need saturation which is determined by what in SPR?

Student: It should be a concentration at least 5 times.

What is a unit? Saturation the first and foremost thing that you do before you start the SPR.

Student: R max.

There you go R max right. So, why should we determine an R max what is the unique property of R max that allows you to interpret your SPR data faithfully?

So, suppose I said I want 400 RU as R max rate I want 400 RU as R max value. I note the molecular weight of the ligand I know the molecular weight of the analyte and what is the another parameter that is in the equation.

Student: Stoichiometric coefficient.

Now tell me why should I go back and calculate what the R max is?

Student: It gives an expected value.

Exactly. So, if I got something weird then what I expected, there are two things that happen right. One there is some weird nonspecific binding going on or two the estimated stoichiometry is not correct. The second you come you interpret later the first thing that you should worry when you get a more R max than what you expected.

Because you know what you immobilized right and if it is one is to one no matter what you do those mathematical equations have to be satisfied. So, whether you understand the kinetics, whether you fit it into a 1 is to 1; binding or more complex binding isotherms the first level of checkpoints have to be done ok. So, when you are doing SPR you be careful about all these things your calculations you first do predetermine calculations. You go back and look at your sensorgram and see whether you have got all these values correct. You estimate the equilibrium dissociation constant by if even if you cant get the kinetics estimate the equilibrium dissociation.

Find that your binding interactions are very similar to what you have obtained by other studies. It is better to do SPR after doing some kind of measurements that tell you the equilibrium constant. Is there any other technique that allows you to look at equilibrium constant any biophysical technique?

Student: ITC.

So, you do ITC, you will get the stoichiometry there yeah its very good for stoichiometric measurements. And then you get the KD there as well and then you combine it with K on and K off; you get a delta s delta g delta h the stoichiometry KD on and off you are done right.

So, that is a way to probe the protein-protein interactions in depth. And, if you have a select example that you started with the hypothesis and it has ended up giving you the expected results then you try and do all these parameters. And, see whether either by screening molecules to find the inhibitor or you start with the structure guided the design by docking. Or you start with already known peptide that it binds and then you begin modifying the peptide using chemists help and then, designing their molecules so, that you can get better and better in a better.

This is the reason why we are doing SPR. And so, far it seems you guys rightly pointed out what are the problems, but what is the what is the positive aspect of this data what is the positive aspect of the data?

Student: You see some binging.

Very good you see some binding and then what happens with the mutants?

Student: They are binding very well.

The mutants behave like they are expected to behave they do not bind very well right. Now you see here its not so, bad we still fitted these binding kinetics and why do I say it is not so, bad?

Student: Chi square value.

Chi square and why is chi square important?

Student: It tells relation between expected and observed value.

What is it that you look for when you look at the chi square expected and observed and what should it look like? Residuals right. So, it is the deviation from the residual that gives you the chi square value this is pretty tight. So, you can believe all these right. So, you look at the Gankyrin and we get K D is around 12 micro molar is it right?

This is something that we expect around 12 to 50 micromolar is what we expect. And the ka and KD and you look at the mutants ok; the mu it seems to suggest that the mutants have better on rate right. And very similar or slightly faster off slower off rate than Gankyrin the wild type. And you look at all these KD measurements and look at the R max despite poor binding they seem to be behaving pretty well in terms of the kinetic constant we do not understand this.

I am showing you what the graph values are, but we do not understand this yeah. We are trying to interpret this as to what may be the problem and definitely we are not happy because if you look at it is not very clear to you from here. It is we are achieving up to 15 RUS on the

binding and which is not very good. And, we are not able to achieve R max because the chip whatever we have used whether it is (Refer Time: 30:47) NTA chips or we have tried direct immobilization we are having problems.

And nonspecific binding to the flow cell is seen. And some of the times many times some of the flow cells do not work. And what we have come to conclude by looking at many many interactions of this kind changing. The chemistry you definitely need to change the chemistry either it is a (Refer Time: 31:09) it is a biotin streptavidin or immobilization or a capture. You need to do multiple things to confirm that these are behaving the way you should.

So, what we have understood now is that the IFC that the fluid excel itself is a problem and that needs a replacement every 6 months costs about 6 lakhs; but we are also trying alternative chemistry as well on this and trying to reverse. So, we took the streptavidin chip and try to buy in the biotin peptide and then come up with the protein what is the advantage that I have in that case? The analyte is bound no that is a biotin related peptide to the streptavidin chip and I am passing the ligand instead of having the ligand immobilized and I pass the analyte which is a short peptide which is better.

Student: Bigger molecule should be immobilized.

Bigger molecule on the chip.

How is the how is the RU generated?

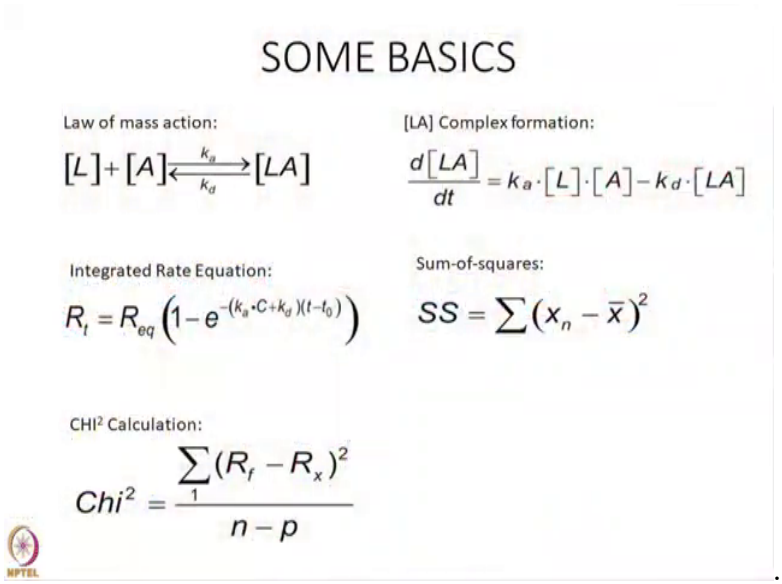
Student: Yes, it is reverse.

Why is it reverse you finish your thought?

Student: So, that you get higher RUs value when the change in mass is greater.

Exactly. So, when you design experiments that you want. So, here is where the sensitivity question of sensitivity comes in. And, if you are looking at very small molecules it is better to have the ligand and then you bind the analyte, but g (Refer Time: 32:21) of t 200 to be able to capable of detecting small molecules. But, once probably we get these things going straight and especially the fluidic cell we should be able to see these things happening, but yes that is the main point.

(Refer Slide Time: 32:36)



And these are before you start a SPR experiment just keep these things in mind, you calculate you find the expected values go back interpret your sensogram. Do not try to fit the data before understanding the sensogram; you can fit it to any equation that you want you will get a value you will get a chi square never ever do that. Most often it is the simple one is to one binding, that is happening just because your sense of you did not do the experiment right. A

second order fit or a third order fit might give you a very bad very good fit because now you are increasing the parameters to fit.

So, the your sensograms will look nicely fitted, but that may be a wrong interpretation. Most often it is the first one is to binding that you should try; if there are after doing many many experiments you think that things are not explained by one is to one binding then you begin to use other equations of this.

So, these are some things I definitely have to look at you have to look at equilibrium. Try to look at and how do I, how do I enhance dissociation can I enhance dissociation?

Student: Changing the pH.

I can change the pH; can I change anything else?

Student: Flow rate flow rate.

Flow rate very nice anything else why should I change p H?

Student: (Refer Time: 34:05).

What happens if I change pH?

Student: Interaction will get weak (Refer Time: 34:07).

Why should the interaction get weak? I change the proteomics right. So, what is the other way of doing it dissociating two proteins?

Students: Salt (Refer Time: 34:17).

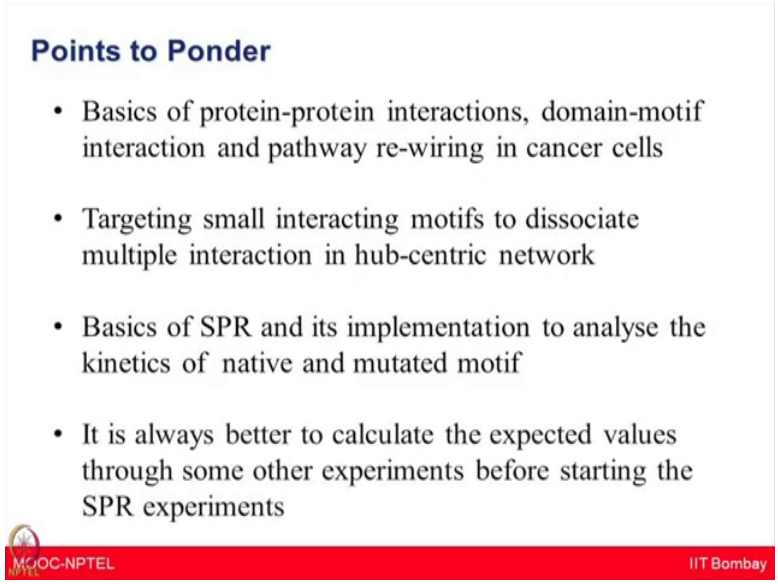
Salt.

Student: (Refer Time: 34:19).

Then I am speaking to experts there was my last site.

Thank you very much.

(Refer Slide Time: 34:26)



So, I hope now you are convinced that SPR based systems is a very powerful platform to generate high quality data, for biomolecular interactions. Especially, to obtain the dissociation constant the KD values on rate off rate, the kinetic data which could provide you very quantitative valuable information.

Thank you.